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Characterization of Physiological Roles and Prognostic Importance of IR/IGF-IR Hybrid Receptors in Breast Cancer

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14. ABSTRACT
An inducible dimerization system will be used to study the IR/IGF-IR Hybrid receptor by constructing chimeric IR and chimeric IGF-IR with different dimerization domains. MCF10A cells stably expressing chimeric IGF-IR were generated and homodimerizer AP20187 treatment was able to induce activation of chimeric IGF-IR and down stream AKT and ERK pathways. Retroviral vectors for chimeric IR with FKBP and FRB domains were constructed and will be used to created stable cell lines for further study. To measure the IR/IGF-IR hybrid receptor on paraffin embedded specimens, a proximity ligation assay was used for dual detection of IR/IGF-IR hybrid receptor using both anti-IR and anti-IGF-IR antibodies. The PLA assay was optimized in both cell pellet and breast tissue specimens to detect IR/IGF-IR hybrid receptors and more studies will be done to confirm the specificity of the PLA assay.

15. SUBJECT TERMS
Insulin Receptor (IR), IGF-IR, IR/IGF-IR Hybrid receptor (Hybrid-R), inducible dimerization, Proximity ligation assay (PLA), breast cancer

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INTRODUCTION

Insulin receptor (IR) mediates the endocrine actions of insulin to regulate glucose homeostasis and metabolism [1]. The IGF signaling cascade is a major regulator of cell proliferation and survival, and thus it is important in many cancers including breast cancer [2-5]. IR/IGF-IR hybrid receptors (Hybrid-R) have been shown to occur when IR and IGF-IR are co-expressed [6]. Moreover, Hybrid-Rs are widely overexpressed in breast cancer specimens compared to normal breast tissue and the Hybrid-R levels are higher than both IR and IGF-IR levels in most breast cancer specimens [7]. However, Hybrid-R signaling and role of Hybrid-R in breast cancer remain un-known due to the difficulty to study the Hybrid-R specifically since they co-exist with IR and IGF-IR. **I hypothesize that Hybrid-Rs play a role in tumor progression by promoting cell proliferation, survival and migration, and that the levels of Hybrid-R may correlate with clinical outcome and biological characteristics of breast cancer patients.** In this study, I will utilize an inducible dimerization system to identify signaling pathways driven by Hybrid-R and develop a new assay that allows us to measure Hybrid-R levels on paraffin embedded specimens then further evaluate the prognostic value of Hybrid-R in breast cancer.

Body

1) Training environment

The Breast Center at BCM is an excellent teaching environment for graduate students and post-docs. In the past year, I have accomplished the following:

- Completed " Biostatistics for clinical and translational research" course
- Completed " Translational Breast Cancer Research" course
- Successfully completed my qualifying exam
- Presented data in poster format at the graduate student symposium at BCM
- Presented data in poster format at Cancer Center Symposium at BCM
- Oral presentation at TBMM seminar
- Oral presentation at Breast Center R&D workshop
- Oral Presentation at Breast Center Trainee Retreat

2) Research Project

In this project, we are utilizing an inducible dimerization system in collaboration with Dr. Muthuswamy from Cold Spring Harbor to generate chimeric IR and chimeric IGF-IR to specifically study Hybrid-R. Chimeric IGF-IR retroviral vector with FKBP domain and empty vectors with FKBP and FRB domain were provided by Dr. Muthuswamy. Homodimerizer AP20187 is used to dimerize chimeric IR or chimeric IGF-IR with FKBP domain and heterodimerizer AP21967 is used to dimerize chimeric IGF-IR with FKBP and chimeric IR with FRB to form Hybrid-R. The principle of inducible dimerization system for this project is attached in the Appendx.

<u>Specific Aim 1:</u> Create breast cancer cell lines stably transfected with inducible receptors and identify signaling pathways and specific adaptor proteins activated by these inducible receptors.

- ***Construct retroviral vectors for chimeric IR***

To create chimeric IR that allows homodimerization, the beta sub-unit of IR was PCR amplified and then inserted into retroviral empty vector with FKBP domain using XbaI enzyme.

To create chimeric IR that allows heterodimerization with chimeric IGF-IR, the beta sub-unit of IR was PCR amplified and then inserted into retroviral empty vector with FRB domain using XbaI enzyme.

- ***Confirm the expression of chimeric IR and chimeric IGF-IR protein expression***

Chimeric IGF-IR vector with FKBP domain was transfected into retrovirus packaging cell line PT67 and cells were harvested after antibiotic selection for stable expression of chimeric IGF-IR. As shown in Fig.1A, chimeric IGF-IR proteins were detected using both IGF-IR and HA antibodies and they have higher molecular weight than endogenous IGF-IR proteins.

Chimeric IR vectors with FKBP and FRB domain were transiently transfected into 293 cells and cells were harvested at 48 hours after transfection for immunoblot analysis. In Fig. 1B, the chimeric IR with FKBP domain was detected using both IR

and HA antibodies and the chim eric IR wit h FRB domain was detected using both I R and Glu-Glu antibodies. Both kind of chimeric IR ran at higher molecular weight than endogenous IR.

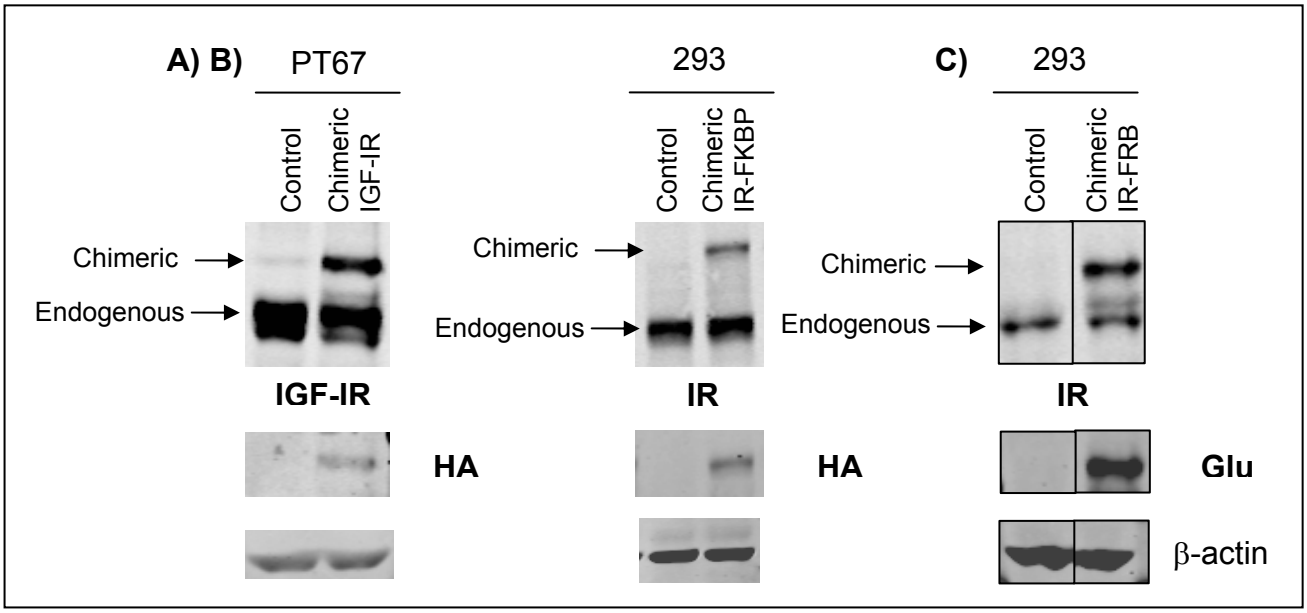


Figure 1. Expressio n of chimeric IR and IGF-IR in transiently transfected 293 and PT67 cells. A) Transfected PT67 cells were harvested after antibiotic selection for stable expression of chimeric IGF-I R. Proteins were harvested in 5% SDS buffer and ana lyzed by western blot for chimeric IGF-IR expression using anti- IGF-IR and anti-HA antibodies. B) and C) 293 ce lls were transiently tra nsfected with chimeric IR with FKBP or FRB domain. P roteins were harvested at 48 hours after transfection for western blot analysis. Anti-IR and anti-HA antibodies were used to detect chimeric IR with FKBP domain and anti-IR and anti-Glu-Glu antibodies were used to detect chimeric IR with FRB domain.

- ***Confirm the activation of chimeric IGF-IR upon dimerizer treatment in MCF10A cells stably expressing chimeric IGF-IR***

I generated retrovirus of chimeric IGF- IR from PT67 cells and infected MCF10A cells. Infected MCF10A cells were then subj ected to Geneticin se lection. Geneticin resistant clones wer e harvested for imm unoblot to confirm t he chimeric IGF-IR expression. To verify that the chimeric IGF-IR are func tional, I performed immunoblot analysis using MCF10A cells stably expres sing chimeric IGF-IR treated with or without homodimerizer AP20187 (Fig. 2) . As expected, treatment of A P20187 successfully induced phosphorylation of chimeric IGF-IR and activation of AKT and ERK.

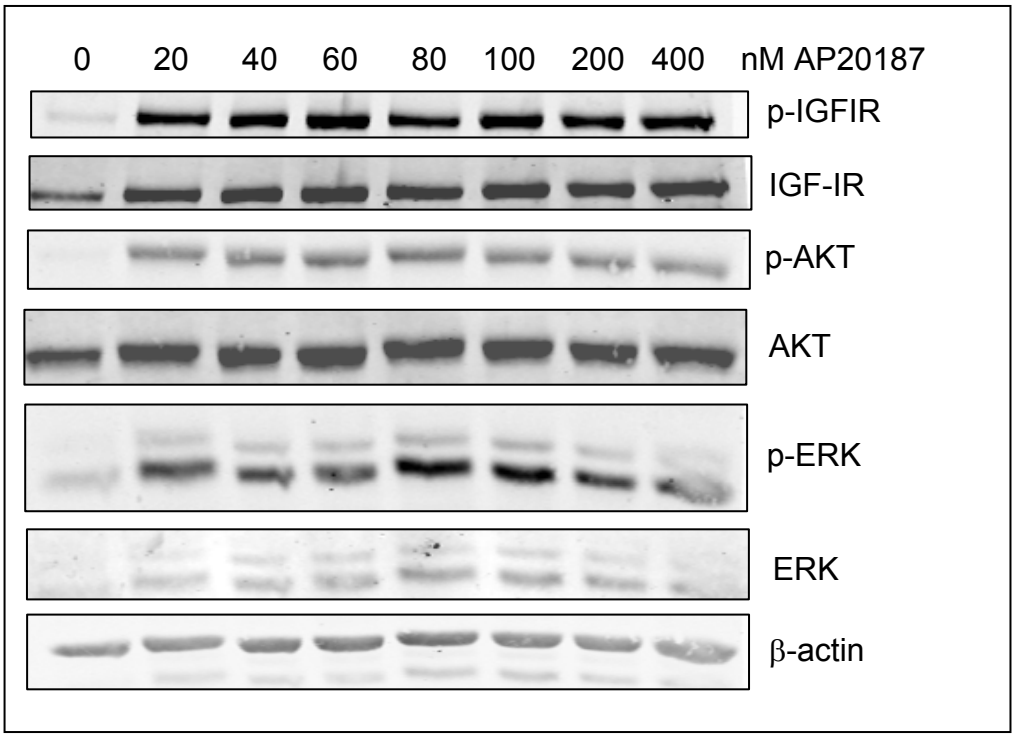


Figure 2. AP20187 induces activation of chimeric IGF-IR and down stream AKT and ERK in MCF10A cells. MCF10A cells stably expressing chimeric IGF-IR with FKBP domain were treated with incremental doses of AP20187 for 15 minutes after serum free starvation overnight. Proteins were detected by western blot using antibodies against p-IGFIR, IGF-IR, p-AKT, AKT, p-ERK, ERK and β-actin.

<u>Specific Aim 3:</u> Determine the levels of IR/IGF-IR hybrid receptors in breast cancer samples and correlate these to clinical characteristics and outcome.

In the proposal, I proposed to use FRET/F LIM technique to measure the Hybrid-R on paraffin embedded samples. However, I enc ountered some difficulties to find the microscope with FLIM capability and it is more difficult to quantify the lost of signal instead of gain of signal. Therefore, I decided to switch to proxim ity ligatio n assay (PLA) developed by Olink Bioscience t o measure the Hybrid-R. The principle of PLA assay is attached in the Appendix.

- ***Create control samples for PLA assay development***

I generated cell pellet controls using si RNA knock down IGF-IR in MDA-MB-134 and MCF7 cells to create samples with different levels of Hybrid-R expression. From immunohistochemistry (IHC) of IGF-IR (Fig. 3), there was significant decrease of signal on IGF-IR siRNA treated cell pellet samples.

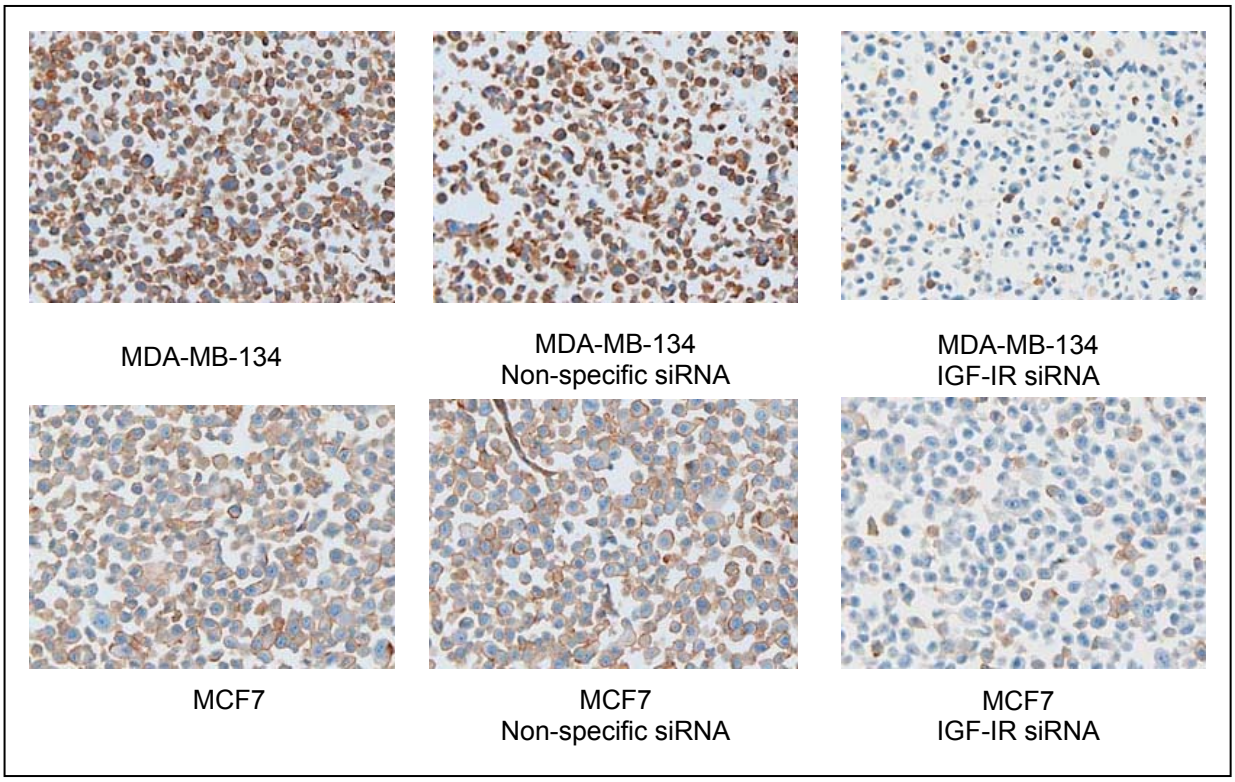


Figure 3. Immunohistochemistry of IGF-IR for MDA-MB-134 and MCF7 cell pellet samples. MDA-MB-134 and MCF7 cells were transiently transfected with non-specific siRNA or IGF-IR siRNA. Cells were scraped down and fixed in 10% formalin for two hours prior embedding in to paraffin block. 5 μm sections were cut from each block and IHC for IGF-IR was performed using anti-IGF-IR antibody from Ventana.

- ***Optimization of PLA assay for Hybrid-R detection***

To test if the antibody pair could be used for Hybrid-R detection in PLA assay, I used sections from MDA-MB-134 cell pellets to perform the PLA assay. In this experiment, I included several negative controls to confirm that the signal is truly from the dual recognition of IR and IGF-IR antibody to the Hybrid-R, not from non-specific binding of probes or IR antibody or IGF-IR antibody alone. As shown in figure 4, there was no signal detected on no antibody control, no IR antibody and no IGF-IR antibody control. In contrast, there were bright red spots (indicated by white arrow in fig. 4) detected on MDA-MB-134 sections while the signal was absent in MDA-MB-134 section with IGF-IR siRNA knock down.

Next, I performed a PLA assay on a Tissue microarray (TMA) slides with normal breast tissue and dead-end breast tumors to determine if the condition for cell pellet controls could also work on tissue samples. As shown in figure 5, the signal pattern observed in cell pellet control was absent in normal breast tissue. However, the red signal (indicated by white arrow in fig. 5) was detected in many, but not all, breast tumor samples on this TMA.

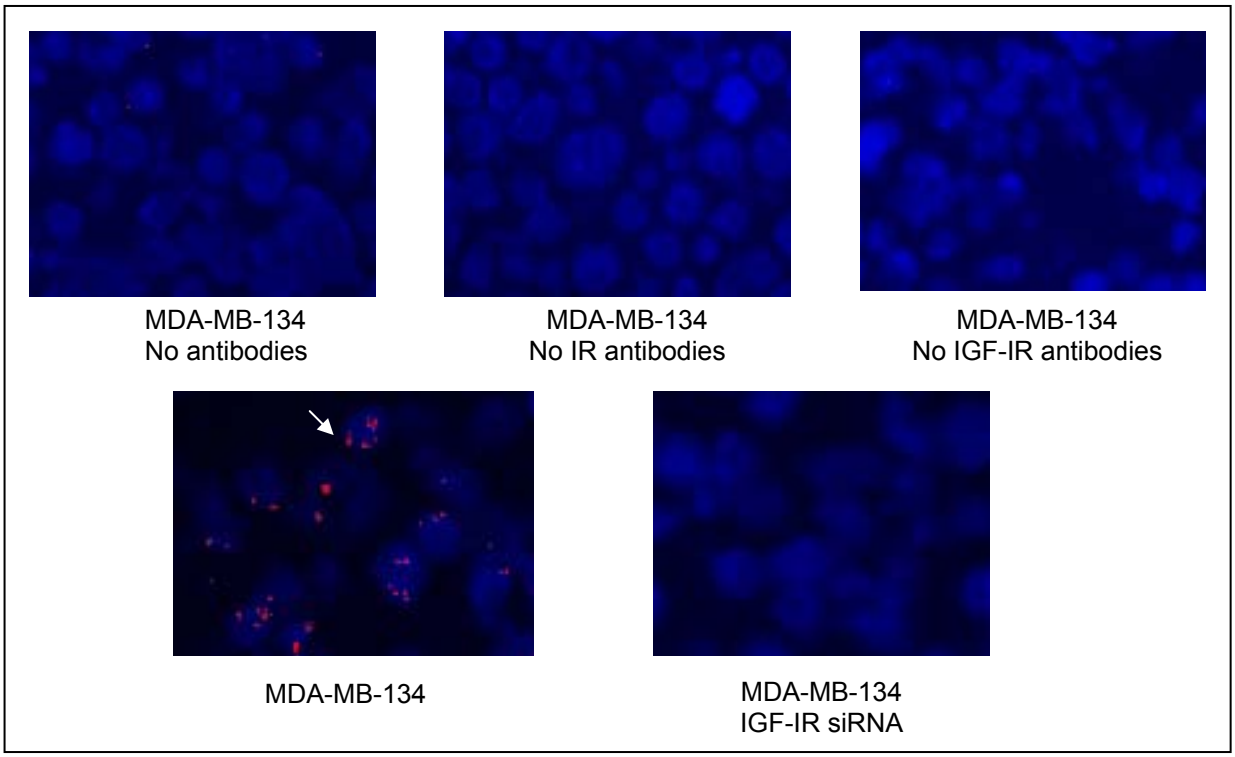


Figure 4. Detection of Hybrid-R on MDA-MB-134 cell pellet samples using PLA assay. MDA-MB-134 cell pellet sections were deparaffinized in Xylene. They were then placed in sodium citrate buffer and cooked for 20 minutes in pressure cooker for antigen retrieval. Blocking buffer provided in the PLA assay kit was used for blocking for 30 minutes. Monoclonal anti-IR antibody from Santa Cruz and Polyclonal anti-IGF-IR antibody from Cell Signaling were used as primary antibody pair for duo recognition of Hybrid-R. The rest of the processes were performed according to the instruction from the PLA assay manual. The images were taken by Nuance system, a multispectral image system developed by CRI.

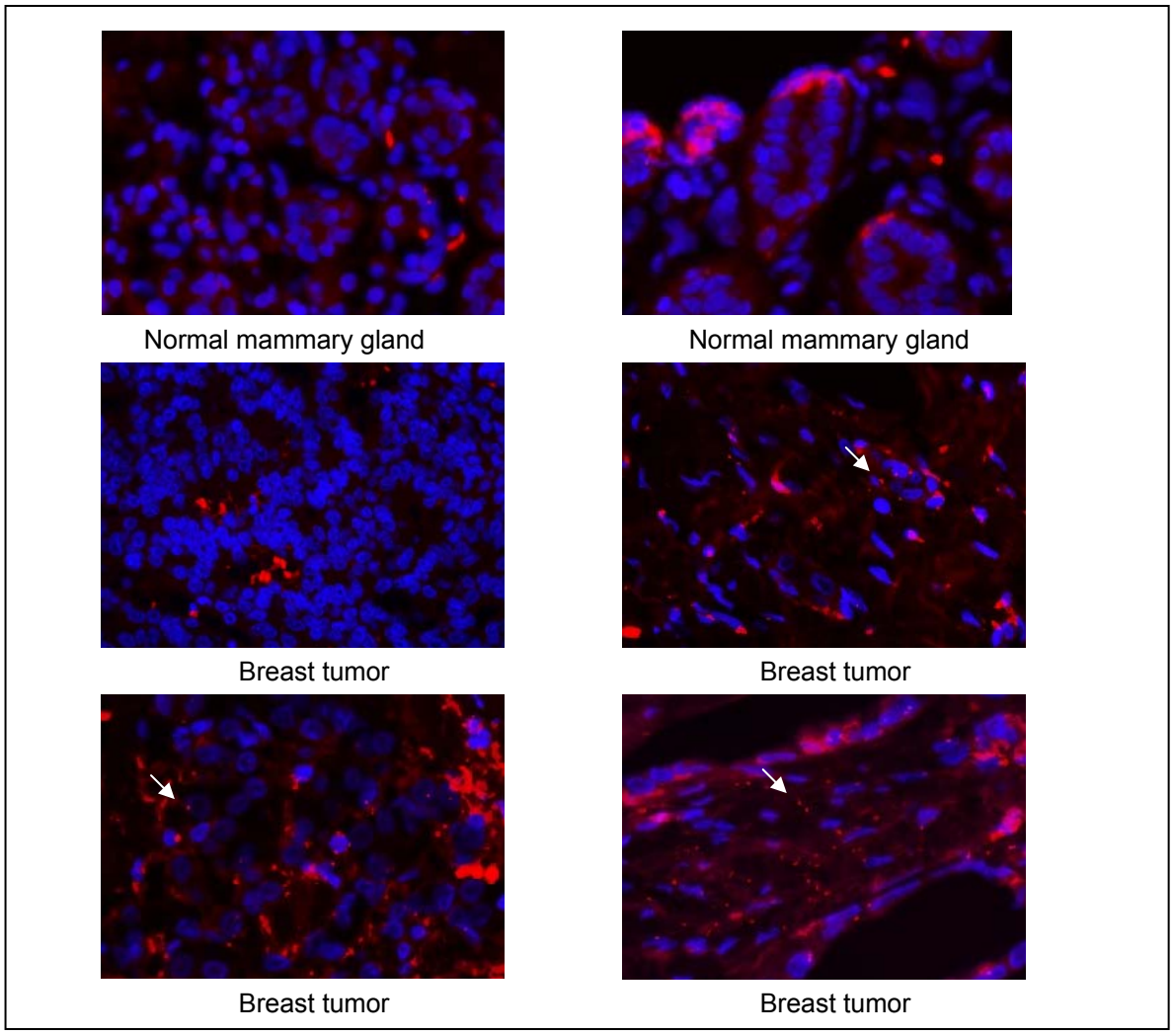


Figure 5. Detection of Hybrid-R on dead end breast tissue samples using PLA assay. A TMA slide with dead-end normal breast tissue and breast tumor specimens from Breast Center pathology core was used to test the PLA assay on tissue specimens. The staining condition used for cell pellet samples was followed in this assay and the images were also acquired using Nuance system.

Key Research Accomplishments

- Confirmed the expression and functionality of chimeric IGF-IR in MCF10A cells.
- Constructed retroviral vector for chimeric IR with FKBP and FRB domains.
- Confirmed the expression of chimeric IR with FKBP and chimeric IR with FRB in transiently transfected 293 cells.
- Optimized PLA assay for IR/IGF-IR Hybrid receptor detection in paraffin embedded specimens.

Reportable Outcomes

N/A

Conclusion

It has been very difficult to specifically st udy the role of IR/IGF -IR Hybrid receptor (Hybrid-R) since it is impo ssible to stimulate endogenous Hybr id-R without activating IR or IGF-IR. By using the inducible dimerization syst em, I will be able to differentiate the effect of Hybrid-R from that of IR and IGF-IR. The next step of th is project is to select candid ate breast cancer cell lines to study the role of Hybrid-R in breast cancer.

There are currently up to fifty different breas t cancer cell lines that represent different subtype of breast cancer. To better understand the action of insulin and IGF in breast cancer, our lab performed a Reverse Phase Prot ein Array (RP PA) expe riment on tw enty different breast cancer cell lines stimulated with insulin or IGF fo r six different time points. We are currently in the process of data analysi s for the RPPA experiment. From the result of RPPA a nalysis, we will be ab le to identify br east cancer cell lines that are insulin a nd IGF responsive and from that, we will have some insights about what cell lines as model to study Hybrid-R.

The results from preliminary experiment for Hybrid-R detection on paraffin embedded samples using PLA as say are promising. To furt her prove that this assa y is really spec ific for Hybrid-R detectio n, I will further create cont rol samples usin g cells sta bly e xpressing chimeric IR and IGF-IR that allows inducibl e Hybrid-R formation. AP21967 treated cells are expected to have increase of signal com pared to vehicle treated cells and this will be a very good positive control for this PLA assay.

References

1. Dupont, J. & LeRoith, D. Insulin and insulin-like growth factor I receptors: similarities and differences in signal transduction. *Horm Res* **55 Suppl 2**, 22-6 (2001).

2. Sachdev, D. & Yee, D. The IGF system and breast cancer. *Endocr Relat Cancer* **8**, 197-209 (2001).

3. LeRoith, D. & Roberts, C.T., Jr. The insulin-like growth factor system and cancer. *Cancer Lett* **195**, 127-37 (2003).

4. Zhang, D., Samani, A.A. & Brodt, P. The role of the IGF-I receptor in the regulation of matrix metalloproteinases, tumor invasion and metastasis. *Horm Metab Res* **35**, 802-8 (2003).

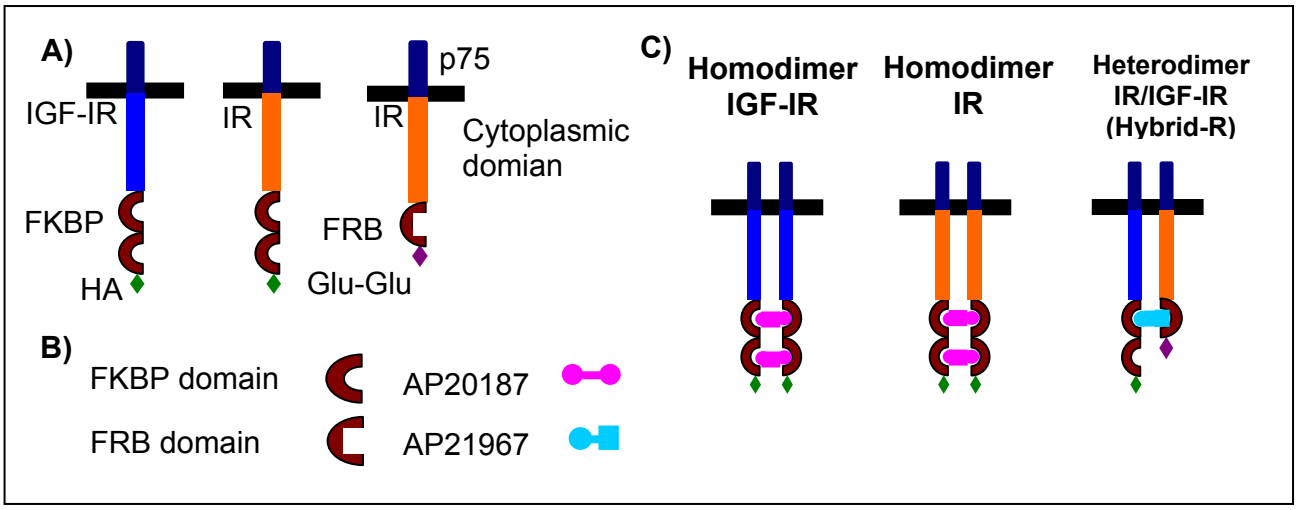
5. Samani, A.A., Yakar, S., LeRoith, D. & Brodt, P. The role of the IGF system in cancer growth and metastasis: overview and recent insights. *Endocr Rev* **28**, 20-47 (2007).

6. Soos, M.A., Whittaker, J., Lammers, R., Ullrich, A. & Siddle, K. Receptors for insulin and insulin-like growth factor-I can form hybrid dimers. Characterisation of hybrid receptors in transfected cells. *Biochem J* **270**, 383-90 (1990).

7. Frasca, F., Pandini, G., Vigneri, R. & Goldfine, I.D. Insulin and hybrid insulin/IGF receptors are major regulators of breast cancer cells. *Breast Dis* **17**, 73-89 (2003).

Appendix

I. Inducible dimerization system



Each chimeric receptor consists of extra-cellular domain of p75 that avoids the ligand binding (insulin, IGF-I and IGF-II) to the chimeric receptor, beta sub-unit of IR or IGF-IR, dimerization domain (FKBP or FRB) and a tag (HA or Glu-Glu). There are three different chimeric receptors in this system: (1) Chimeric IGF-IR with FKBP domain tagged with HA (2) Chimeric IR with FKBP domain tagged with HA (3) Chimeric IR with FRB domain tagged with Glu-Glu. There are two kinds of dimerizers: AP20187 that brings two FKBP domains together for homodimerization and AP21967 that brings one FKBP and one FRB domain together for heterodimerization.

II. Principle of PLA assay (from Duolink PLA manual by Olink Bioscience)

Typical starting materials are adherent cells, cytospin preparations or tissue sections on a glass slide, fixed, pre-treated and blocked with a blocking reagent according to the requirements of the primary antibodies used.

1: The samples are incubated with primary antibodies that bind to the protein(s) to be detected.

2: Secondary antibodies conjugated with oligonucleotides (PLA probe MINUS and PLA probe PLUS) are added to the reaction and incubated.

3: The Hybridization solution, consisting of two oligonucleotides (illustrated as red bands), is added and the oligonucleotides will hybridize to the two PLA probes if they are in close proximity.

4: The Ligation solution is added together with Ligase (yellow), joining the two hybridized oligonucleotides to a closed circle.

5: The Amplification solution, consisting of nucleotides (not shown) is added together with Polymerase (yellow). The oligonucleotide arm of one of the PLA probes acts as a primer for a rolling-circle amplification (RCA) reaction using the ligated circle as a template, generating a concatemeric (repeated sequence) product extending from the oligonucleotide arm of the PLA probe.

6: The Detection solution, consisting of fluorescently labeled oligonucleotides, is added and the labeled oligonucleotides will hybridize to the RCA product. The signal is easily visible as a distinct fluorescent dot and analyzed by fluorescence microscopy.